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ORIGINAL PAPER

Irradiation leads to sensitization of hepatocytes to TNF-α-mediated apoptosis by upregulation of IκB expression

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Abstract This study aimed to reveal the pathophysiological signalling responsible for radiation-induced sensitization of hepatocytes to TNF-α-mediated apoptosis. IκB was upregulated in irradiated hepatocytes. Administration of IκB antisense oligonucleotides prior to irradiation inhibited occurrence of apoptosis after TNF-α administration. Caspases-8, -9 and -3 activities were increased in irradiated hepatocytes and downregulation of apoptosis by IκB antisense oligonucleotides was mediated by suppression of caspases-9 and -3 activation but not of caspase-8 activation, suggesting that radiation-induced sensitization of hepatocytes to TNF-α-mediated apoptosis additionally requires changes upstream of caspase-8 activation. Herein, upregulation of FLIP may play a crucial role. Cleavage of bid, upregulation of bax, downregulation of bcl-2 and release of cytochrome c after TNF-α-administration depend on radiation-induced upregulation of IκB, thus demonstrating an apoptosis permitting effect of IκB.

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Introduction

It is well accepted that hepatocytes are quite radioresistant compared to other cells [1–7]. On the other hand, the liver is a highly radiosensitive organ: the development of radiation-induced liver disease (RILD) is considered to be a major dose limiting complication in abdominal irradiation [8]. The threshold dose for whole liver irradiation is assumed to be between 20 and 30 Gy [9]. Hepatic vein lesions and parenchymal cell death are the most prominent histological lesions [10]. Furthermore, liver irradiation above the threshold dose is not followed by a recovery phase and restitution ad integrum, but leads to progressive liver fibrosis and cirrhosis at least in animal studies [11].

The pathophysiological mechanisms of hepatocellular cell death after irradiation are widely unknown. In fact, in our previous publication we were able to demonstrate that irradiation alone does not lead to apoptosis of hepatocytes. However, irradiation leads to susceptibility of hepatocytes to TNF-α-mediated apoptosis, whereas incubation of non-irradiated hepatocytes with TNF-α does not lead to apoptosis [12]. The intracellular mechanisms underlying these effects are still unclear. In previous experiments, we detected early changes in gene expression after irradiation of hepatocytes in vitro by cDNA gene array expression analysis [13]. In these experiments we used samples of hepatocytes from two different isolations. Concerning IkB, in the first sample an upregulation could be observed, while in the second it was less than 1.5-fold enhanced compared to sham-irradiated control hepatocytes. The data on IkB were not included in [13] because of the contrasting result. In the present study, we were able to show that upregulation of IκB is crucial for the induction of susceptibility of irradiated hepatocytes to TNF-α-mediated apoptosis.

Additionally, further changes upstream of caspase 8 activation are also required.

Materials and methods

Animals

Male Wistar rats (200–260 g) were kept on a 12-h day/night rhythm (light from 07:00 to 19:00 h) with free access to water and food. Rats were anaesthetized with pentobarbital (60 mg kg^{-1} body weight) before preparation of hepatocytes between 08:00 and 09:00 h. The study protocols were approved by a government review board. All animals received care in compliance with institutional guidelines, the German Convention for Protection of Animals and the National Institutes of Health guidelines.

Cell culture

Hepatocytes were isolated by collagenase perfusion. Cells (1×10^6 per dish) were maintained under standard conditions [16% O_2, 79% N_2, and 5% CO_2 (by volume)] in DMEM (Biochrom, Berlin, Germany) containing 0.5 nM insulin added as a growth factor for culture maintenance, 100 nM dexamethasone required as a permissive hormone, and 10% foetal calf serum.

Irradiation

Hepatocytes on the first day after isolation were irradiated with 6 MV photons at a dose rate of 2.4 Gy min^{-1} using a Varian Clinac 600 C accelerator (Varian, Palo Alto, USA).

Flow cytometric and fluorescence microscopic quantification of living, apoptotic and necrotic hepatocytes

For quantification of apoptotic cells, we used flow cytometry after trypsination of hepatocytes (Epics ML, Coulter, Krefeld, Germany). To detect apoptotic, changes staining with Annexin V-FITC/propidium iodide and the TUNEL method (Tdt-mediated X-dUTP nick end labelling) were used (Boehringer, Mannheim, Germany). Data obtained by TUNEL-labelling were identical to those obtained with the Annexin V-FITC/propidium iodide binding. As a third method to detect apoptosis on the single-cell level, we used the mitochondrial membrane sensor kit from Clontech, Palo Alto, California, USA. Briefly, this method is based on the fact that the dye is able to accumulate in intact mitochondria. In case of apoptosis, it aggregates in the cytosol and changes its colour from red to green. By this change of the fluorescence colour, it is possible to discriminate between apoptotic and living cells.

Western blot analysis of IκB, Bcl-2, Bid, Bax, cytochrome c, caspase-3, caspase-9, and caspase-8, FLIP

Cells were harvested at different time points after plating, lysed in hot Laemmli buffer (95°C) and processed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to Laemmli [14]. The protein content of cellular lysates was calculated by the Coomassie Protein Assay (Pierce, Rockford, IL). Proteins were transferred onto Hybond-ECL nitrocellulose hybridization transfer membranes according to Towbin et al. [15]. Immunodetection was performed according to the ECL Western blotting protocol. Antibodies against IκB (Calbiochem, Frankfurt, Germany), Bcl-2 (Santa Cruz, Heidelberg, Germany), Bid (Santa Cruz, Heidelberg, Germany) and Bax (Calbiochem, Frankfurt, Germany), caspase-8 (Calbiochem, Frankfurt, Germany), caspase-9 (Calbiochem, Frankfurt, Germany), caspase-3 (Calbiochem, Frankfurt, Germany), FLIP (Upstate, Cambridge, UK) were used at 2.5 μg ml^{-1} solutions, and peroxidase-labelled anti-mouse and anti-rabbit immunoglobulins were each used at a 1/1,000 dilution. To detect cytochrome c released into the cytoplasm, cells were gently pelletted by low speed centrifugation ($300 \times g$), washed once in PBS, pelletted again, and resuspended in 100 μl of 10 mM HEPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, and 10% sucrose. Samples were incubated on ice for 30 min, transferred to a Dounce homogenizer, and lysed by three strokes with a B type pestle. The solution was transferred to a 1.5 ml tube and centrifuged ($300 \times g$) for 10 min at 25°C. The supernatant was transferred to a fresh tube and frozen on crushed dry ice for 1 min, followed by high speed centrifugation (14,000g) for 10 min to pellet the heavy membrane fraction and mitochondria. The supernatant was removed and represented the mitochondria-depleted cytoplasmic fraction, which was then immunoblotted for reactivity with anti-cytochrome c antibody as described above. Homogeneous loading was assured by Western Blot analysis of β-actin (Sigma, Deisenhofen, Germany) in each experiment.

Nuclear Extracts Electromobility Shift Assay (EMSA) and Super Shift Assay

Nuclear extracts were prepared according to the method of Dignan et al. [16]. Hepatocytes (5×10^6) were harvested by scrape-harvesting into TBS (20 mM Tris, pH 7.2, 0.15 M NaCl). The cells were resuspended in buffer A (0.2 ml; 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, leupeptin and aprotinin each 1 μg ml^{-1}, PMSF 0.5 mM, 1 mM orthovanadate, 2 mM pyrophosphate) and incubated 15 min on ice. After that, 25 ml 2.5% NP-40/buffer A was added, mixed by inversion and the nuclei

pelleted (500 g, 4 min, 4°C). The nuclear proteins were extracted and analyzed as described previously. The protein concentration was determined with Coomassie Assay (Pierce). The labelling was done with T4 polynucleotide kinase. The antibodies specific for NFκB p65 were purchased from Santa Cruz Biotechology (Santa Cruz Californis). EMSA (NFkB) and supershift (NFkB) reactions were done in 20 ml reaction mixture based on the extraction buffer with NaCl containing 10 mg nuclear extract 10^5 cpm labelled oligonucleotides and, in the case of supershift, 4 mg of rabbit polyclonal antibody against the p65-NFκB (Transcruz, Santa Cruz Biotechnology). The binding reaction was performed at 4°C overnight. DNA–protein complexes were resolved by electrophoresis through a 4% polyacrylamide gel containing 45 mM TRIS–borate and 1 mM ethylene diamine tetraacetic acid (EDTA) at pH 8.0. Gel was dried and exposed to X-ray film overnight. To detect unspecific binding, competition experiments with a 100-fold excess of unlabelled specific oligonucleotides were performed. In all control experiments, no unspecific binding was detected.

Inhibition of IκB translation by antisense technique

In order to inhibit IκB translation, we used an IκB antisense kit (Biognostics, Goettingen, Germany). A FITC-labelled randomized-sequence phosphorothioate oligonucleotide was used to monitor uptake efficiency. An uptake of the oligonucleotide into 80% of the cells was achieved within 8 h as could be shown by immunofluorescence microscopy. Subsequently, hepatocytes were irradiated. Occurrence of apoptosis was measured using the TUNEL-method.

Caspase-8, caspase-3, and caspase-9 activity

For detection of active caspase-8, caspase-3, or caspase-9, we used the Active Caspase Set (Pharmingen, Germany). Cell lysates of 5×10^6 cells were applied and active caspases-8, -3 or active caspase-9 was detected according to the manufacturer's protocol. To evaluate substrate specificity, caspase-3, caspase-8, and caspase-9 inhibitors (Oncogene, MA, USA) were used.

Statistical analysis

Results are expressed as mean + SD. After assessing normal distribution of the data, significance in differences was tested by ANOVA, followed by Bonferroni's post (hoc) test, and $P < 0.05$ was taken to imply statistical significance.

Results

IκB and NFκB-expression after irradiation of hepatocytes

We could show an upregulation of IκB in rat hepatocytes after irradiation with 8 Gy compared to sham-irradiated cells 6, 12 and 24 h after irradiation on protein level (Fig. 1a). As expected, active NFκB is downregulated at the same time as could be shown by EMSA (Fig. 1b).

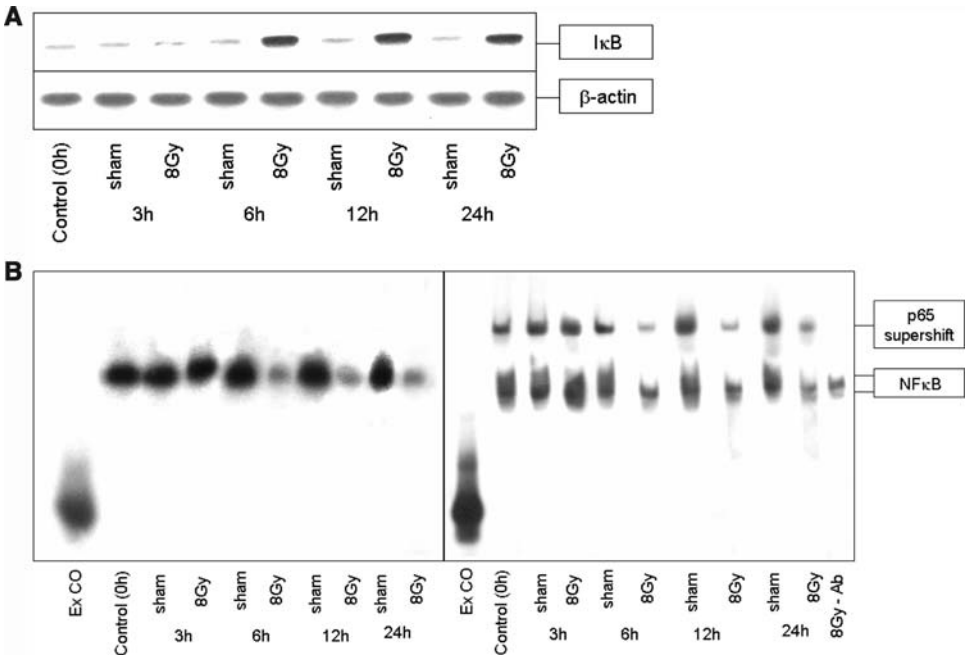

Fig. 1 **a** Western Blot analysis of IκB expression at different time points after irradiation with 8 Gy or sham irradiation. The blot presented is an example of samples of five different isolations. **b** NFκB EMSA (*left*) and supershift assay (*right*). Nuclear extracts from hepatocytes (sham irradiated or irradiated with 8 Gy) at different time points were incubated with a ^{32}P-labelled consensus NFκB oligonucleotide with or without antibodies to NFκB p65 isoform. Gels are representative for three independent experiments from three different isolations; ExCO = 100-fold excess cold NFκB oligonucleotide; 8 Gy-Ab = EMSA without NFκB p65 supershift antibody

Susceptibility of rat hepatocytes to TNF-α-mediated apoptosis following irradiation

Administration of 100 U ml^{-1} TNF-α 6 h after irradiation led to an increment of apoptosis occurrence in hepatocyte cultures by 28.6% (8 Gy) or 57.1% (25 Gy) within 24 h after irradiation and by 62.5% (8 Gy) or 175% (25 Gy) within 48 h after irradiation (Fig. 2a). This increment of portion of apoptotic hepatocytes could also be observed when TNF-α was administrated 12 h after irradiation. By this means, an increase of apoptotic cell portion could be observed by 120% (8 Gy), respectively 200% (25 Gy) 24 h after irradiation, and by 150% (8 Gy) or 317% (25 Gy) 48 h after irradiation (Fig. 2b). However, when TNF-α was added to the culture medium 18 h after irradiation, no effect on apoptosis of irradiated hepatocytes could be observed suggesting that susceptibility of irradiated hepatocytes to TNF-α-mediated apoptosis only occurs within a certain time window after irradiation (Fig. 2c). However, since downregulation of NFκB–DNA-binding activity does not depend on the time point of TNF-α-administration [6 or 18 h after irradiation (Fig. 2d)] additional irradiation-induced intracellular changes seem to be necessary for the development of susceptibility of irradiated hepatocytes to TNF-α-induced apoptosis.

Effect of IκB-antisense on susceptibility of irradiated hepatocytes to TNF-α-mediated apoptosis

In order to investigate, whether the radiation-induced upregulation of IκB influences susceptibility of rat hepatocytes to TNF-α-mediated apoptosis, we preincubated hepatocytes with IκB-antisense oligonucleotides. Subsequently, the hepatocyte cultures were irradiated with 8 Gy. By this means, IκB expression was drastically downregulated as could be shown 24 and 48 h after irradiation (Fig. 3a). Regarding active NFκB, TNF-α leads to an upregulation in non-irradiated hepatocytes. Irradiation not only leads to downregulation of active NFκB but also completely impairs the up-regulatory effect of TNF-α (Figs. 2d, 3b). However, by means of IκB antisense oligonucleotides, the active NFκB is again present in higher amounts in irradiated hepatocytes and a further up-regulatory effect of TNF-α could be observed (Fig. 3b). Control oligonucleotides had no effect on apoptosis of irradiated hepatocytes or on irradiated hepatocytes, to which TNF-α was added 6 h after irradiation. However, irradiated cultures, which were preincubated with IκB-antisense oligonucleotides, lost their susceptibility to TNF-α-mediated apoptosis. In fact, the increment of apoptotic cells due to TNF-α-administration 6 h after irradiation was reduced to control levels at the time points 24 and 48 h after irradiation (Fig. 3c).

Influence of IκB-antisense oligonucleotides on activity of caspase-8, caspase-9, and caspase-3 in rat hepatocytes irradiated with 8 Gy with/without TNF-α administration

To further elucidate the interaction between TNF-α-signalling and involvement of radiation-dependent upregulation of IκB for TNF-α mediated apoptosis of hepatocytes, activity of caspase-8, caspase-9, and the effector caspase-3 was measured. Independent from IκB-expression, TNF-α (administered 6 h after irradiation with 8 Gy) led to an increase of caspase-8 activity up to 3.2-fold within 24 h and up to 4.4-fold within 48 h after irradiation (Fig. 4a). In the same settings, TNF-α also led to a strong increase of caspase-9 activity (4.9-fold within 24 h and 6.2-fold within 48 h after irradiation). However, this TNF-α-induced increase of caspase-9 activity in irradiated hepatocytes could fully be blocked to control level when IκB-antisense oligonucleotides were used (Fig. 4b). This effect of IκB-antisense oligonucleotides was carried forward also for the activity of the effector caspase-3 (Fig. 4c), and in all cases Western blot analyses supported the caspase activity assays. On the other hand, it has also to be mentioned that administration of TNF-α to non-irradiated hepatocytes did not lead to an increased activity of caspase-8, caspase-9, or caspase-3 (data not shown).

Effect of irradiation and IκB-antisense oligonucleotides on Bid, Bcl-2, Bax and cytochrome c release

As demonstrated above, activation of caspase-9 seems to be crucial for TNF-α mediated apoptosis induction of irradiated hepatocytes, suggesting the involvement of the mitochondrial apoptosis pathway. In fact, administration of TNF-α to irradiated hepatocytes leads to cleavage of Bid to tBid. This effect could be cancelled by administration of IκB-antisense oligonucleotides. Furthermore, a TNF-α mediated downregulation of anti-apoptotic Bcl-2 and upregulation of pro-apoptotic Bax could be observed, which also could be avoided by usage of IκB-antisense oligonucleotides. Finally, in the same setting a significant release of cytochrome c into the cytosol could be observed (Fig. 5a). These data strongly support the thesis that radiation-induced upregulation of IκB leads to susceptibility of hepatocytes to TNF-α-mediated apoptosis by the mitochondrial apoptosis pathway. This thesis is further supported using the mitochondrial membrane sensor kit to detect apoptosis. This method allows to detect apoptosis-related changes at the level of the mitochondrial membrane in irradiated hepatocytes as early as 12 h after administration of TNF-α (Fig. 5b). At this point of time, no significant increase of apoptosis could be observed at the level of DNA damage as measured by the TUNEL method or at the level of the cell membrane (AnnexinV/propidium iodide-

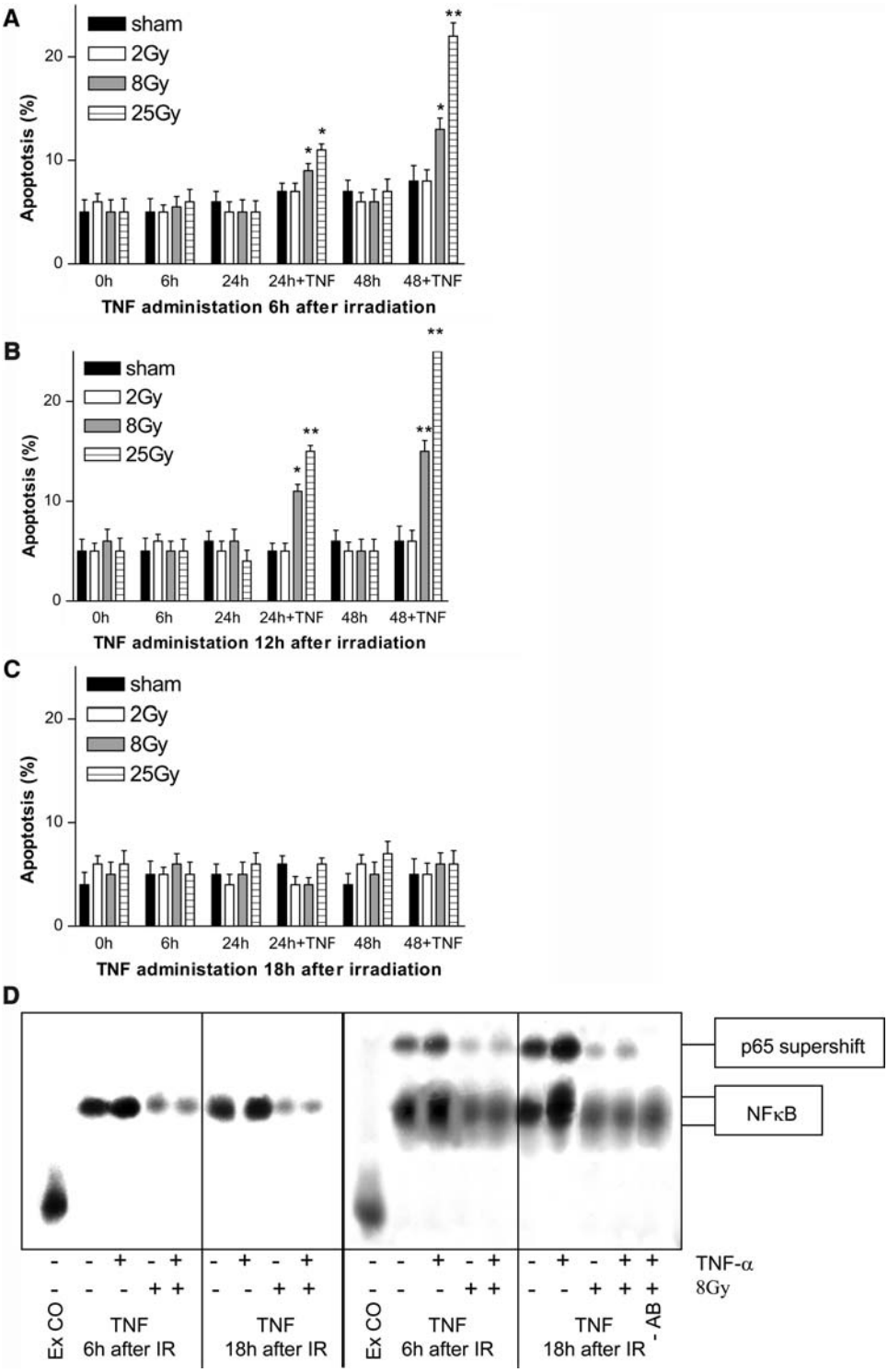

Fig. 2 Apoptosis of hepatocytes at different time points after irradiation (sham, 2, 8, 25 Gy) with or without TNF-α (100 U ml^{-1}) administrated 6 h (**a**), 12 h (**b**) or 18 h (**c**) after irradiation or sham irradiation. Apoptosis rates were assessed using the AnnexinV/PI- method. Values presented are mean + SD of seven different hepatocyte isolations. Level of significance: * P<0.05; ** P < 0.01. **d** NFκB EMSA (*left*) and supershift assay (*right*). Nuclear extracts from hepatocytes (sham irradiated or irradiated with 8 Gy at time point (48 h after irradiation) were incubated with a ^{32}P-labelled consensus NFκB oligonucleotide with or without antibodies to NFκB p65 isoform. 100 U ml^{-1} TNF-α was added 6 or 18 h after irradiation/sham irradiation. Gels are representative of three independent experiments from three different isolations; *ExCO* 100-fold excess cold NFκB oligonucleotide; *-AB* EMSA without NFκB p65 supershift antibody

method). Using the latter methods, increment of apoptosis could first be observed 18 h after TNF-α administration (24 h after irradiation). Notably, pre-treatment with IkB-antisense oligonucleotides was capable to prevent irradiated hepatocytes from apoptotic changes. It is however, still unclear, why TNF-α leads to activation of caspase-8 after irradiation, which cannot be influenced by means of IκB-downregulation and does not lead to caspase-8 activation in sham-irradiated hepatocytes. Western blot analysis revealed that FLIP, a potent inhibitor of caspase-8 activation, is downregulated after irradiation and its expression is not under the control of IκB, suggesting that downregulation of FLIP due to irradiation might enable the pro-apoptotic TNF-α-signal-transduction pathway (Fig. 5c).

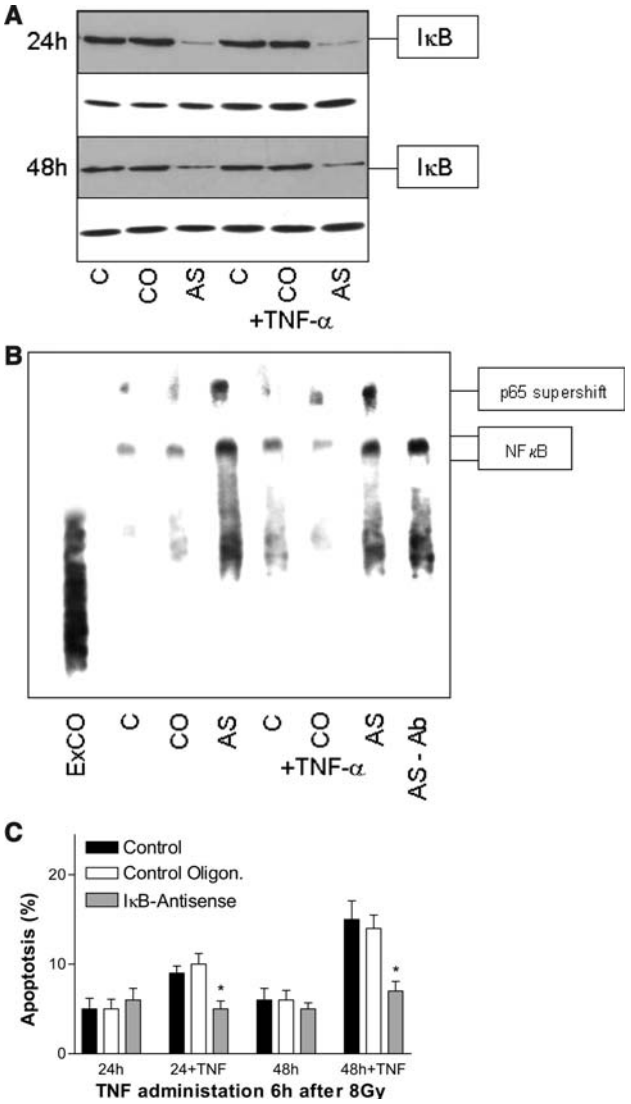

Fig. 3 **a** Western blot analysis of hepatocytes incubated with or without IκB antisense nucleotide 12 h before irradiation (8 Gy) with or without TNF-α (100 U ml^{-1} 6 h after irradiation). Cells were harvested 24 or 48 h after irradiation. We could confirm the data in five different blots of five different isolations. *C* Control; *CO* control oligonucleotide; *AS* antisense oligonucleotide. **b** NFκB supershift assay. Nuclear extracts from hepatocytes incubated with or without IκB antisense nucleotide 12 h before irradiation with or without TNF-α (100 U ml^{-1} 6 h after irradiation). Cells, harvested 48 h after irradiation, were incubated with a ^{32}P-labelled consensus NFκB oligonucleotide and antibodies to NFκB p65 isoform. Gel is representative of three independent experiments from three different isolations; *ExCO* 100-fold excess cold NFκB oligonucleotide; *AS-Ab* probe of nuclear extracts from cells incubated with IκB antisense oligonucleotides 12 h prior to irradiation and administration of TNF-α 6 h after irradiation without NFκB p65 supershift antibody. **c** Apoptosis induction in irradiated hepatocytes with or without preincubation with IkB-antisense oligonucleotides with or without TNF-α (100 U ml^{-1}) 6 h after irradiation. Cells were harvested 24 or 48 h after irradiation and apoptosis was measured using the AnnexinV/PI- method. Mean + SD of seven different hepatocyte isolations. Level of significance: * $P < 0.05$

Discussion

NFκB is an essential component of ionizing radiation-triggered signal transduction pathways that can lead either to cell death or survival, depending on the respective cell type (for a review see [17]), and numerous studies have demonstrated that inhibition of NFκB by different means increased sensitivity of cancer cells to the apoptotic action of diverse effectors such as TNF-α, chemo- or radiotherapies (for review see [18]). In both primary rat hepatocytes and a non-transformed rat hepatocyte cell line, inhibition of NFκB-activity by adenoviral delivery of a IκB superrepressor sensitized these cells to death from TNF-α [19, 20]. However, TNF-α was also recently shown to sensitize for DNA-damage-induced apoptosis via an NF-kappa-B independent mechanism [21].

We show that blocking IκB-transcription by IκB-antisense oligonucleotides in hepatocytes and thus preventing the radiation-induced upregulation of IκB sensitizes hepatocytes to TNF-α mediated apoptosis. These data suggest that inactivation of NFκB is crucial for inducing susceptibility of these cells to TNF-α-mediated apoptosis.

Based on the existence of one or two distinct domains, the TNF-R superfamily members are divided into two subgroups, the death domain (DD)-containing receptors and the TNF-R-associated factor (TRAF) interacting receptors [22]. TNF-R1 belongs to the first group and the earliest adaptor molecule recruited to the intracellular DD of the TNF-R1 is the TNF-R-associated death domain (TRADD) [23]. The apoptotic cell death pathway is activated following recruitment of the fas-associated death domain (FADD/MORT1) protein to TRADD through interactions between the DD in each protein [24]. FADD carries a second domain, the death effector domain (DED), which recruits proteins from the caspase family of enzymes. Upon co-localization with FADD, high, localized concentrations of procaspase-8 undergo autoproteolytic cleavage, releasing activated caspase-8. This complex has been termed death-inducing signalling complex (DISC) [25, 26]. After DISC formation, TNF-induced hepatocyte death results from the mitochondrial death pathway in which caspase-8 activation leads to functional changes in the mitochondria, such as mitochondrial permeability transition resulting in the release of mitochondrial proteins (e.g. cytochrome c) into the cytosol. The mechanism of cytochrome c release involves cleavage of the Bcl-2 family member Bid (tBid) [27]. Truncated Bid (tBid) migrates into the mitochondria and triggers oligomerization of the pro-apoptotic Bcl-2 members Bax and Bak. These molecules then insert into the mitochondrial membrane, which results in release of mitochondrial proteins including cyto-

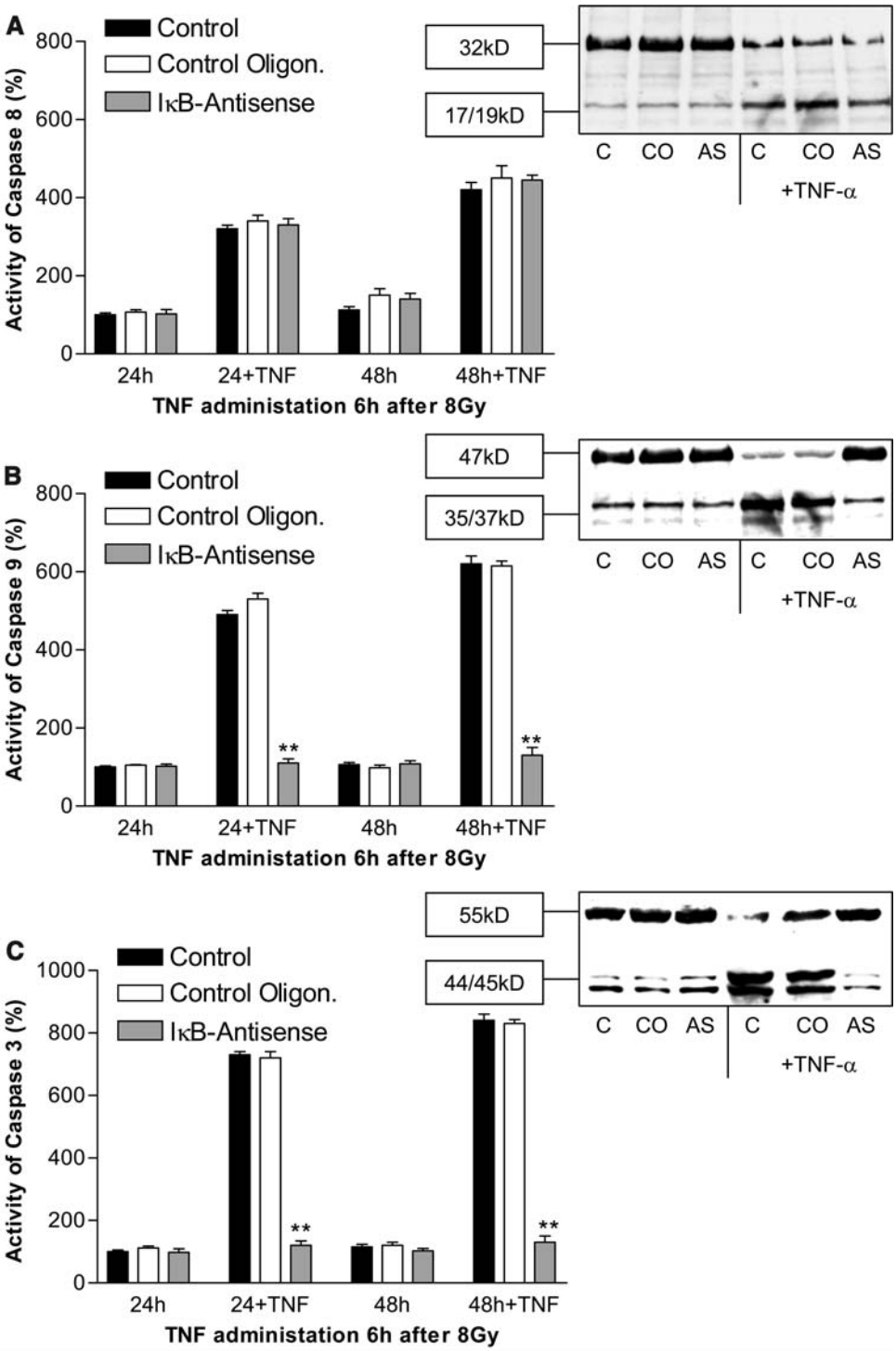

Fig. 4 Activity of caspase-8 (Fig. 3a), -9 (Fig. 3b), and -3 (Fig. 3c). After a 12 h incubation with antisense oligonucleotides, hepatocytes were irradiated with 8 Gy (time point 0 h). Cells were harvested 24 or 48 h after irradiation with or without TNF-α administration 6 h after irradiation. Indicated are mean + SD of seven different hepatocyte isolations. Level of significance: ** $P < 0.01$. In all cases, Western blot analysis (*insets*) supports the activity assays demonstrating cleavage of the respective pro-caspases into their active forms. The Blots presented, derived from cell lysates of hepatocytes irradiated with 8 Gy and harvested 48 h after irradiation with or without TNF-α administration 6 h after irradiation. *C* Control; *CO* control oligonucleotide; *AS* antisense oligonucleotide)

chrome c [28]. Following release into the cytosol, cytochrome c triggers formation of the apoptosome, a complex with apoptosis protease activating factor-1 (APAF-1) and procaspase-9. Caspase-9 becomes activated and in turn activates caspase-3, resulting in apoptosis [29]. Cell types that are dependent on this mitochondrial death pathway have been termed type II cells. In contrast, type I cells generate high levels of caspase-8 that directly activate caspase-3. Since activation of caspase-8 due to TNF-α administration after irradiation only leads to caspase-3 activation when NFκB-activity is downregulated, whereas high activity of NFκB as a result of usage of IκB-antisense oligonucleotides inhibits caspase-9 but also caspase-3 activity, our data are in accordance with those terming hepatocytes to be type II cells [30, 31]. This may also be supported by the data demonstrating cleavage of Bid,

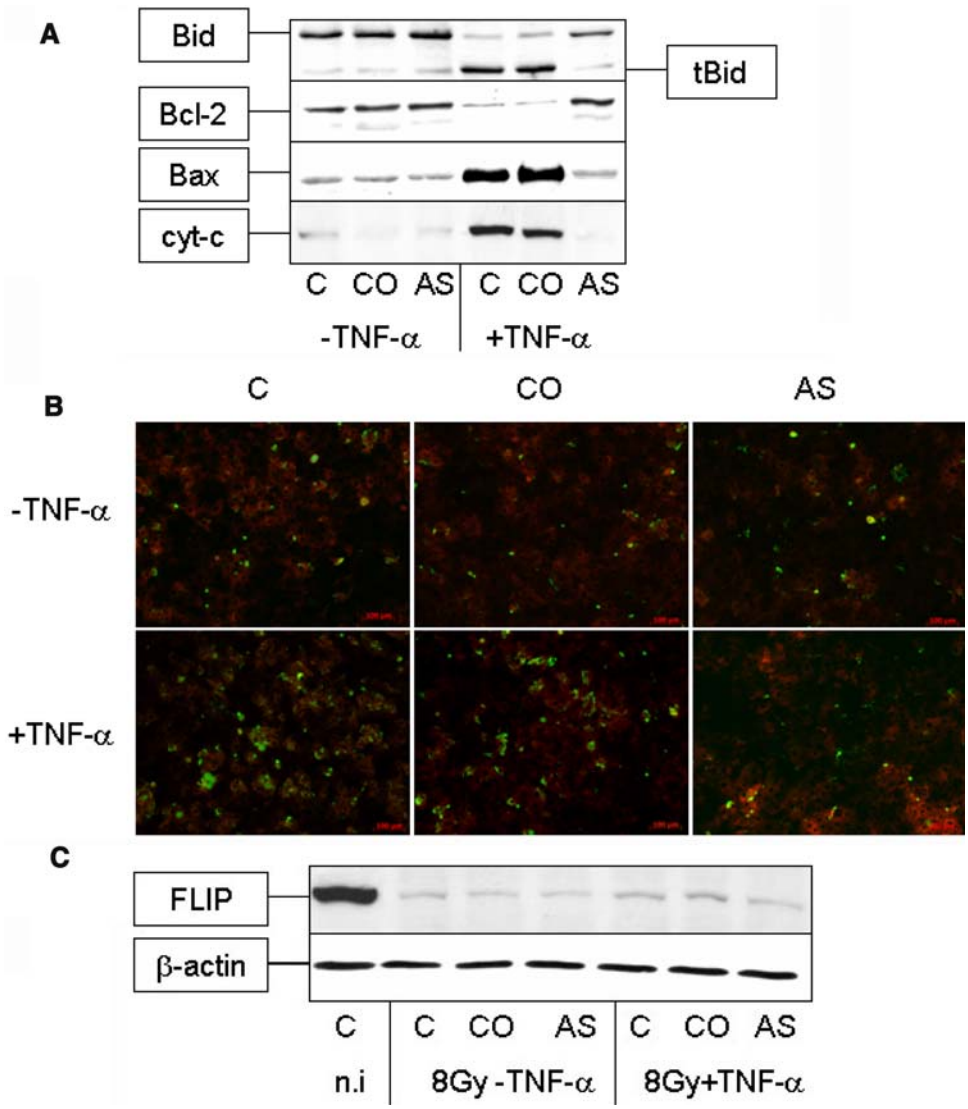

Fig. 5 **a** Western blot analysis of Bid, Bcl-2, Bax in cell lysates as well as cytosolic cytochrome c. After 12 h incubation with IκB-antisense oligonucleotides, hepatocytes were irradiated with 8 Gy (time point 0 h). Subsequently, cells were treated with or without TNF-α 6 h after irradiation and cells were harvested 48 h after irradiation. *C* Control; *CO* control oligonucleotide; *AS* antisense oligonucleotide. We could confirm the data in five independent experiments. **b** Hepatocyte apoptosis detection by the mitochondrial membrane sensor kit. In healthy cells, the dye accumulates in the mitochondria and fluoresces red. In case of apoptosis, the dye accumulates in the cytoplasm and fluoresces green. *C* Control; *CO* control oligonucleotide; *AS* antisense oligonucleotide). **c** Expression of FLIP in hepatocytes. After 12 h incubation with IκB-antisense oligonucleotides, hepatocytes were irradiated with 8 Gy (time point 0 h). Subsequently, cells were treated with or without TNF-α 6 h after irradiation and cells were harvested 48 h after irradiation. Data of non-irradiated (n.i.) and irradiated cultures are given. *C* Control; *CO* control oligonucleotide; *AS* antisense oligonucleotide)

upregulation of Bax, downregulation of Bcl-2, and release of cytochrome c into the cytosol dependent on upregulation of IκB in irradiated hepatocytes. The mechanism how NFκB-signalling influences TNF-α death signalling pathway in hepatocytes is unknown. However, our data from irradiated hepatocytes demonstrate that NFκB is capable to block TNF-α-mediated apoptosis in hepatocytes downstream of caspase-8 activation but upstream of caspase-9 activation. Besides upregulating IκB and thereby facilitating activation of caspase-9 by TNF-α, irradiation also enables TNF-α-mediated caspase-8 activation in rat hepatocytes. Noteworthy, this part of TNF-α signalling after irradiation is not under the control of NFκB suggesting that irradiation induces additional changes at the level of DISC-formation and caspase-8 activation. One possibility might be the downregulation of apoptosis inhibiting proteins in the TNF-α signal transduction pathway like FLIP (fas ligand inhibitory protein), which we were able to demonstrate in our system [32, 33], members of the SOCS-family (silencers of cytokine signalling) [34] and/or members of the IAP-family (inhibitor of apoptosis) [35, 36]. However, preliminary Western blot data on IAP did not show any regulation of IAP after irradiation when compared to non-irradiated hepatocytes. A rapid re-production of these proteins after radiation-induced downregulation might be the reason, why susceptibility of hepatocytes after irradiation to TNF-α-mediated apoptosis only occurs when TNF-α is administered within a time window of 6–12 h after irradiation, whereas the effect of irradiation on IκB-upregulation and consecutively on downregulation of active NFκB lasts at least 48 h. However, our data also show that induction of the TNF-α-death pathway within this time window is capable of maintaining the apoptosis process beyond this vulnerable period of time suggesting that TNF-signalling itself may lead to downregulation of apoptosis inhibitory proteins.

In a recent paper by Huang et al. [37], the authors show that mice pre-treated with antisense oligonucleotides for TNFR1 when compared to control mice show less radiation-induced liver damage as measured by increment of transaminases and by the TUNEL method, which supports our thesis that TNF-α released by Kupffer cells and/or macrophages due to irradiation finally may lead to apoptosis of hepatocytes via the pathway described above. Huang et al. [37] also demonstrated that pre-treatment of their mice with antisense oligonucleotides for FAS did not prevent radiation-induced liver apoptosis. In our system however, using FASL (CD95L) in a similar approach as used for TNF-α, apoptosis of hepatocytes could be observed (data not shown). This apparent contradiction may be resolved when regarding the time window of observation. Huang et al. observed a time period of 8 h. In our previous paper, we reported that already within 3 h post-irradiation we could find a pronounced increment of TNF-α in the liver. Therefore, induction of apoptosis in hepatocytes may occur by the pathway described above. However, the ligand for FAS (CD95L) necessary to induce apoptosis is mainly carried by inflammatory cells such as macrophages. Therefore, the time of observation by Huang et al. may be to short, since within this time the migration of inflammatory cells through the sinusoidal epithelial cell layer may not occur and thus considerable contact between CD95L carrying cells and the CD95 carrying hepatocytes does not happen. From our data on TNF-α and CD95L-induced apoptosis of hepatocytes, both leading to activation of caspase-8 in irradiated hepatocytes but not in control cells we would suggest that activation of death domain containing receptors leading to caspase-8 activation like FAS, TNFR1, and probably also the TRAIL receptors are candidates to induce hepatocyte apoptosis after liver irradiation. Similarly, it has been shown that the CD95/CD95–ligand system mediates radiation-induced pneumonitis and TRAIL receptor antibodies can enhance radiation effects on tumour cells [38, 39].

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